

## DNA ENCODING CANINE VON WILLEBRAND FACTOR AND METHODS OF USE

### RELATED APPLICATIONS

The present invention is a continuation-in-part of U.S. Serial No. 08/896,449,  
5 filed July 18, 1997, which claims priority from U.S. Serial No. 60/020,998, filed July  
19, 1996, both hereby expressly incorporated by reference.

### FIELD OF THE INVENTION

This invention relates generally to canine von Willebrand factor (vWF), and  
more particularly, to the gene encoding vWF as well as a genetic defect that causes  
10 canine von Willebrand's disease.

### BACKGROUND OF THE INVENTION

In both dogs and humans, von Willebrand's disease (vWD) is a bleeding  
disorder of variable severity that results from a quantitative or qualitative defect in von  
Willebrand factor (vWF) (Ginsburg, D. et al., *Blood* 79:2507-2519 (1992); Ruggeri,  
15 Z.M., et al., *FASEB J* 7:308-316 (1993); Dodds, W.J., *Mod Vet Pract* 681-686 (1984);  
Johnson, G.S. et al., *JAVMA* 176:1261-1263 (1988); Brooks, M., *Probl In Vet Med*  
4:636-646 (1992)). This clotting factor has two known functions, stabilization of Factor  
VIII (hemophilic factor A) in the blood, and aiding the adhesion of platelets to the  
subendothelium, which allows them to provide hemostasis more effectively. If the  
20 factor is missing or defective, the patient, whether human or dog, may bleed severely.

The disease is the most common hereditary bleeding disorder in both species,  
and is genetically and clinically heterogenous. Three clinical types, called 1, 2, and  
3 (formerly I, II, and III; see Sadler, J.E. et al., *Blood* 84:676-679 (1994) for  
nomenclature changes), have been described. Type 1 vWD is inherited in a dominant,  
25 incompletely penetrant fashion. Bleeding appears to be due to the reduced level of  
vWF rather than a qualitative difference. Although this is the most common form of  
vWD found in most mammals, and can cause serious bleeding problems, it is  
generally less severe than the other two types. In addition, a relatively inexpensive  
vasopressin analog (DDAVP) can help alleviate symptoms (Kraus, K.H. et al., *Vet*  
30 *Surg* 18:102-109 (1989)).

Two other forms of vWD are known, type 2 and type 3. Type 2 is inherited in an autosomal recessive fashion (Ruggeri, Z.M. et al., *FASEB J* 7:308-316 (1993); Brooks, M., *Probl In Vet Med* 4:636-646 (1992)). This type is

also inherited in a dominant fashion and has only rarely been described in dogs (Turrentine, M.A., et al., *Vet Clin North Am Small Anim Pract* 18:275 (1988)).

Type 3 vWD is the most severe form of the disease. It is inherited as an autosomal recessive trait, and affected individuals have no detectable vWF in their blood. Serious bleeding episodes require transfusions of blood or cryoprecipitate to supply the missing vWF. Heterozygous carriers have moderately reduced factor concentrations, but generally appear to have normal hemostasis.

Scottish terriers have Type 3 vWD (Dodds, W.J., *Mod Vet Pract* 681-686 (1984); Johnson, G.S. et al., *JAVMA* 176:1261-1263 (1988)). Homozygotes have no detectable vWF and have a severe bleeding disorder. Heterozygotes have reduced levels of the factor, and are clinically normal (Brooks, M. et al., *JAVMA* 200:1123-1127 (1992)). The prevalence of vWD among Scottish terriers including both heterozygotes and homozygotes has been variously estimated from 27-31% (Stokol, T. et al., *Res. Vet. Sci.* 59:152-155 (1995); Brooks, M., *Proc. 9th ACVIM Forum* 89-91 (1991)).

Currently, detection of affected and carrier Scottish terrier dogs is done by vWF antigen testing (Benson, R.E. et al., *Am J Vet Res* 44:399-403 (1983); Stokol, T. et al., *Res. Vet. Sci.* 59:152-155 (1995)) or by coagulation assays (Rosborough, T.K. et al., *J. Lab. Clin. Med.* 96:47-56 (1980); Read, M.S. et al., *J. Lab. Clin. Med.* 101:74-82 (1983)). These procedures yield variable results, as the protein-based tests can be influenced by such things as sample collection, sample handling, estrous, pregnancy, vaccination, age, and hypothyroidism (Strauss, H.S. et al., *New Eng J Med* 269:1251-1252 (1963); Bloom, A.L., *Mayo Clin Proc* 66:743-751 (1991); Stirling, Y. et al., *Thromb Haemostasis* 52:176-182 (1984); Mansell, P.D. et al., *Br. Vet. J.* 148:329-337 (1992); Avgeris, S. et al., *JAVMA* 196:921-924 (1990); Panciera, D.P. et al., *JAVMA* 205:1550-1553 (1994)). Thus, for example, a dog that tests within the normal range on one day, can test within the carrier range on another day. It is therefore difficult for breeders to use this information.

It would thus be desirable to provide the nucleic acid sequence encoding canine vWF. It would also be desirable to provide the genetic defect responsible for

the defective vWF gene based on the nucleic acid sequence of the normal and defective vWF gene.

### SUMMARY OF THE INVENTION

The present invention provides a novel purified and isolated nucleic acid sequence encoding canine vWF. Nucleic acid sequences containing the mutations that cause vWD in Scottish terriers, Doberman pinschers, Shetland sheepdogs, Manchester terriers and Poodles are also provided. The nucleic acid sequences of the present invention may be used in methods for detecting carriers of the mutation that causes vWD. Such methods may be used by breeders to reduce the frequency of the disease-causing allele and the incidence of disease. In addition, the nucleic acid sequence of the canine vWF provided herein may be used to determine the genetic defect that causes vWD in other breeds as well as other species.

Additional objects, advantages, and features of the present invention will become apparent from the following description, taken in conjunction with the accompanying drawings.

### BRIEF DESCRIPTION OF THE DRAWINGS

The various advantages of the present invention will become apparent to one skilled in the art by reading the following specification and by referencing the following drawings in which:

Figures 1A-1C is the nucleic acid sequence of the canine von Willebrand factor of the present invention (SEQ ID NO: 1);

Figures 2A-2C is a comparison of the human and canine prepro-von Willebrand factor amino acid sequences (SEQ ID NO: 2);

Figure 3 provides nucleotide sequencing ladders for the von Willebrand's disease mutation region for normal (clear), carrier, and affected Scottish terriers, the sequences being obtained directly from PCR products derived from genomic DNAs in exon 4;

Figure 4 illustrates the results of a method of the present invention used to detect the Scottish terrier vWD mutation (SEQ ID NOS: 3-13);

Figure 5 shows the Scottish terrier pedigree, which in turn illustrates segregation of the mutant and normal vWF alleles;

Figure 6 is an illustration showing the splice site comparison between normal

and mutant alleles, and the results of a splice site analysis performed on DNA from the mutant allele (SEQ ID NO: 18);

Figure 8 is a photograph of an agarose gel showing representative results of RFLP analysis of DNA from normal and mutant alleles.

Figure 9 is a histogram of genotypes versus reported vWF values;

Figure 10 is a photograph of a sequencing gel showing the mutation region between a vWD affected and a homozygous normal Shetland sheepdog (SEQ ID NOS: 19 and 20);

5        Figure 11 is a diagram illustrating the *Mwo* I diagnostic test for the Shetland sheepdog Type 3 vWD mutation (SEQ ID NOS: 21-25); and

Figure 12 is a photograph of an agarose gel showing the results of the diagnostic test for the Shetland sheepdog Type 3 vWD mutation.

#### **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

10        The cDNA encoding canine von Willebrand Factor (vWF) has been sequenced, and is set forth in Figures 1A-1C and SEQ ID NO: 1. The deduced amino acid sequence is set forth in Figures 2A-2C and SEQ ID NO: 2. In one embodiment, the mutation of the normal vWF gene which causes von Willebrand's Disease (vWD) in Scottish terriers, a deletion at codon 88 of the normal gene resulting in a frameshift,  
15        is provided. In another embodiment, a splice junction mutation at nucleotide position 7639 of the normal gene, which causes vWD in Doberman pinschers, Manchester terriers and Poodles, is provided. In yet another embodiment, a single base deletion at nucleotide position 937 of the normal gene, causing vWD in Shetland sheepdogs, is provided. The nucleic acid sequences of the present invention may be used in  
20        methods for detecting homozygous and heterozygous carriers of the defective vWF gene.

In a preferred method of detecting the presence of the von Willebrand allele in canines, DNA samples are first collected by relatively noninvasive techniques, *i.e.*, DNA samples are obtained with minimal penetration into body tissues of the animals  
25        to be tested. Common noninvasive tissue sample collection methods may be used and include withdrawing buccal cells via cheek swabs and withdrawing blood samples. Following isolation of the DNA by standard techniques, PCR is performed on the DNA utilizing pre-designed primers that produce enzyme restriction sites on those DNA samples that harbor the defective gene. Treatment of the amplified DNA with  
30        appropriate restriction enzymes such as *Bse*I thus allows one to analyze for the

presence of the defective gene. The present invention is particularly useful for detecting vWD in Shetland sheepdogs, Manchester terriers and Poodles, but to other breeds such as Dutch Kooikers, as well.

The presence of the von Willebrand allele in canines can also be detected utilizing ligation amplification reaction technology (LAR) known to those skilled in the art. LAR is a method analogous to PCR for DNA amplification wherein ligases are employed for elongation in place of polymerases used for PCR. Another alternate  
5 method for detecting the presence of the canine von Willebrand allele also known to those skilled in the art, is allele specific oligonucleotide hybridization, wherein an oligonucleotide of about 20 bp containing the contiguous nucleotides of the allele of interest is hybridized to the canine DNA.

The present invention provides breeders with an accurate, definitive test  
10 whereby the undesired, defective vWF gene may be eliminated from breeding lines. The current tests used by breeders are protein-based, and as noted previously, the primary difficulty with this type of test is the variability of results due to a variety of factors. The ultimate result of such variability is that an inordinate number of animals fall into an ambiguous grouping whereby carriers and noncarriers cannot be reliably  
15 distinguished. The present invention obviates the inherent limitations of protein-based tests by detecting the genetic mutation which causes vWD. As described in the Specific Examples, the methods of the present invention provide an accurate test for distinguishing noncarriers, homozygous carriers and heterozygous carriers of the defective vWF gene.

20 It will be appreciated that because the vWF cDNA of the present invention is substantially homologous to vWF cDNA throughout the canine species, the nucleic acid sequences of the present invention may be used to detect DNA mutations in other breeds as well. In addition, the canine vWF sequence presented herein potentially in combination with the established human sequence (Genbank Accession  
25 No. X04385, Bonthron, D. et al., *Nucleic Acids Res.* 14:7125-7128 (1986); Mancuso, D.J. et al., *Biochemistry* 30:253-269 (1989); Meyer, D. et al., *Throm Haemostasis* 70:99-104 (1993)), may be used to facilitate sequencing of the vWF gene and genetic defects causing vWD, in other mammalian species e.g., by using cross-species PCR methods known by those skilled in the art.

30 It is also within the contemplation of this invention that the isolated and purified

an appropriate host cell, either eukaryotic (e.g., mammalian) or prokaryotic (e.g., *E. coli*). Such DNA may involve alternate nucleic acid forms, such as cDNA, gDNA, and RNA prepared by methods known to those skilled in the art.

accompanied by additional regulatory elements, such as promoters, operators and regulators, which are necessary and/or may enhance the expression of the vWF gene product. In this way, cells may be induced to over-express the vWF gene, thereby generating desired amounts of the target vWF protein. It is further contemplated that  
5 the canine vWF polypeptide sequence of the present invention may be utilized to manufacture canine vWF using standard synthetic methods.

One skilled in the art will appreciate that the defective protein encoded by the defective vWF gene of the present invention may also be of use in formulating a complementary diagnostic test for canine vWD that may provide further data in  
10 establishing the presence of the defective allele. Thus, production of the defective vWF polypeptide, either through expression in transformed host cells as described above for the active vWF polypeptide or through chemical synthesis, is also contemplated by the present invention.

The term "gene" as referred herein means a nucleic acid which encodes a  
15 protein product. The term "nucleic acid" refers to a linear array of nucleotides and nucleosides, such as genomic DNA, cDNA and DNA prepared by partial or total chemical synthesis from nucleotides. The term "encoding" means that the nucleic acid may be transcribed and translated into the desired polypeptide. "Polypeptide" refers to amino acid sequences which comprise both full-length proteins and  
20 fragments thereof. "Mutation" as referred to herein includes any alteration in a nucleic acid sequence including, but not limited to, deletions, substitutions and additions.

As referred to herein, the term "capable of hybridizing under high stringency conditions" means annealing a strand of DNA complementary to the DNA of interest under highly stringent conditions. Likewise, "capable of hybridizing under low  
25 stringency conditions" refers to annealing a strand of DNA complementary to the DNA of interest under low stringency conditions. In the present invention, hybridizing under either high or low stringency conditions would involve hybridizing a nucleic acid sequence (e.g., the complementary sequence to SEQ ID NO: 1 or portion thereof), with a second target nucleic acid sequence. "High stringency conditions" for the  
30 annealing process may involve, for example, high temperature and/or low salt content.

Low stringency conditions may include, for example, lower temperature and/or higher salt concentration than that of high stringency conditions. Such conditions allow for two DNA strands to anneal if substantial, though not near complete complementarity exists between the two. Thus, the present invention may be used to identify sequences



## 15

**Isolation of RNA.** The source of the RNA was a uterus from a Scottish Terrier affected with vWD (factor level < 0.1% and a clinical bleeder), that was surgically removed because of infection. Spleen tissue was obtained from a Doberman pinscher affected with vWD that died from dilated cardiomyopathy (factor level 7% and a clinical bleeder). Total RNA was extracted from the tissues using Trizol (Life Technologies, Gaithersburg, MD). The integrity of the RNA was assessed by agarose gel electrophoresis.

1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, 2025, 2026, 2027, 2028, 2029, 2030, 2031, 2032, 2033, 2034, 2035, 2036, 2037, 2038, 2039, 2040, 2041, 2042, 2043, 2044, 2045, 2046, 2047, 2048, 2049, 2050, 2051, 2052, 2053, 2054, 2055, 2056, 2057, 2058, 2059, 2060, 2061, 2062, 2063, 2064, 2065, 2066, 2067, 2068, 2069, 2070, 2071, 2072, 2073, 2074, 2075, 2076, 2077, 2078, 2079, 2080, 2081, 2082, 2083, 2084, 2085, 2086, 2087, 2088, 2089, 2090, 2091, 2092, 2093, 2094, 2095, 2096, 2097, 2098, 2099, 2100, 2101, 2102, 2103, 2104, 2105, 2106, 2107, 2108, 2109, 2110, 2111, 2112, 2113, 2114, 2115, 2116, 2117, 2118, 2119, 2120, 2121, 2122, 2123, 2124, 2125, 2126, 2127, 2128, 2129, 2130, 2131, 2132, 2133, 2134, 2135, 2136, 2137, 2138, 2139, 2140, 2141, 2142, 2143, 2144, 2145, 2146, 2147, 2148, 2149, 2150, 2151, 2152, 2153, 2154, 2155, 2156, 2157, 2158, 2159, 2160, 2161, 2162, 2163, 2164, 2165, 2166, 2167, 2168, 2169, 2170, 2171, 2172, 2173, 2174, 2175, 2176, 2177, 2178, 2179, 2180, 2181, 2182, 2183, 2184, 2185, 2186, 2187, 2188, 2189, 2190, 2191, 2192, 2193, 2194, 2195, 2196, 2197, 2198, 2199, 2200, 2201, 2202, 2203, 2204, 2205, 2206, 2207, 2208, 2209, 2210, 2211, 2212, 2213, 2214, 2215, 2216, 2217, 2218, 2219, 2220, 2221, 2222, 2223, 2224, 2225, 2226, 2227, 2228, 2229, 2230, 2231, 2232, 2233, 2234, 2235, 2236, 2237, 2238, 2239, 2240, 2241, 2242, 2243, 2244, 2245, 2246, 2247, 2248, 2249, 2250, 2251, 2252, 2253, 2254, 2255, 2256, 2257, 2258, 2259, 2260, 2261, 2262, 2263, 2264, 2265, 2266, 2267, 2268, 2269, 2270, 2271, 2272, 2273, 2274, 2275, 2276, 2277, 2278, 2279, 2280, 2281, 2282, 2283, 2284, 2285, 2286, 2287, 2288, 2289, 2290, 2291, 2292, 2293, 2294, 2295, 2296, 2297, 2298, 2299, 2300, 2301, 2302, 2303, 2304, 2305, 2306, 2307, 2308, 2309, 2310, 2311, 2312, 2313, 2314, 2315, 2316, 2317, 2318, 2319, 2320, 2321, 2322, 2323, 2324, 2325, 2326, 2327, 2328, 2329, 2330, 2331, 2332, 2333, 2334, 2335, 2336, 2337, 2338, 2339, 2340, 2341, 2342, 2343, 2344, 2345, 2346, 2347, 2348, 2349, 2350, 2351, 2352, 2353, 2354, 2355, 2356, 2357, 2358, 2359, 2360, 2361, 2362, 2363, 2364, 2365, 2366, 2367, 2368, 2369, 2370, 2371, 2372, 2373, 2374, 2375, 2376, 2377, 2378, 2379, 2380, 2381, 2382, 2383, 2384, 2385, 2386, 2387, 2388, 2389, 2390, 2391, 2392, 2393, 2394, 2395, 2396, 2397, 2398, 2399, 2400, 2401, 2402, 2403, 2404, 2405, 2406, 2407, 2408, 2409, 2410, 2411, 2412, 2413, 2414, 2415, 2416, 2417, 2418, 2419, 2420, 2421, 2422, 2423, 2424, 2425, 2426, 2427, 2428, 2429, 2430, 2431, 2432, 2433, 2434, 2435, 2436, 2437, 2438, 2439, 2440, 2441, 2442, 2443, 2444, 2445, 2446, 2447, 2448, 2449, 2450, 2451, 2452, 2453, 2454, 2455, 2456, 2457, 2458, 2459, 2460, 2461, 2462, 2463, 2464, 2465, 2466, 2467, 2468, 2469, 2470, 2471, 2472, 2473, 2474, 2475, 2476, 2477, 2478, 2479, 2480, 2481, 2482, 2483, 2484, 2485, 2486, 2487, 2488, 2489, 2490, 2491, 2492, 2493, 2494, 2495, 2496, 2497, 2498, 2499, 2500, 2501, 2502, 2503, 2504, 2505, 2506, 2507, 2508, 2509, 2510, 2511, 2512, 2513, 2514, 2515, 2516, 2517, 2518, 2519, 2520, 2521, 2522, 2523, 2524, 2525, 2526, 2527, 2528, 2529, 2530, 2531, 2532, 2533, 2534, 2535, 2536, 2537, 2538, 2539, 2540, 2541, 2542, 2543, 2544, 2545, 2546, 2547, 2548, 2549, 2550, 2551, 2552, 2553, 2554, 2555, 2556, 2557, 2558, 2559, 2560, 2561, 2562, 2563, 2564, 2565, 2566, 2567, 2568, 2569, 2570, 2571, 2572, 2573, 2574, 2575, 2576, 2577, 2578, 2579, 2580, 2581, 2582, 2583, 2584, 2585, 2586, 2587, 2588, 2589, 2590, 2591, 2592, 2593, 2594, 2595, 2596, 2597, 2598, 2599, 2600, 2601, 2602, 2603, 2604, 2605, 2606, 2607, 2608, 2609, 2610, 2611, 2612, 2613, 2614, 2615, 2616, 2617, 2618, 2619, 2620, 2621, 2622, 2623, 2624, 2625, 2626, 2627, 2628, 2629, 2630, 2631, 2632, 2633, 2634, 2635, 2636, 2637, 2638, 2639, 2640, 2641, 2642, 2643, 2644, 2645, 2646, 2647, 2648, 2649, 2650, 2651, 2652, 2653, 2654, 2655, 2656, 2657, 2658, 2659, 2660, 2661, 2662, 2663, 2664, 2665, 2666, 2667, 2668, 2669, 2670, 2671, 2672, 2673, 2674, 2675, 2676, 2677, 2678, 26

**Reverse Transcriptase-PCR** Total RNA was reverse transcribed using random primers (Bergenhem, N.C.H. et al., *PNAS (USA)* 89:8789-8802 (1992)). The

**DNA Sequence Analysis.** Amplification products of the predicted sizes were isolated from agarose gels by adsorption onto silica gel particles using the manufacturer's method (Qiagen, Chatsworth, CA). Sequences were determined using <sup>33</sup>P-5' end-labeled primers and a cycle sequencing kit (United States Biochemical Corp., Cleveland, OH). The sequences of the 5' and 3' untranslated regions were determined after amplification using Marathon™ RACE kits (Clontech, Palo Alto, CA). Sequences were aligned using the Eugene software analysis package (Lark Technologies, Houston, TX). The sequence of the canine intron four was determined from PCR-amplified genomic DNA.

10       **Design of a Diagnostic Test.** PCR mutagenesis was used to create diagnostic and control *Bst*E I and *Sau*96 I restriction enzyme sites for the test. Amplification conditions for the test are: 94°C, 1 min, 61°C, 1 min, and 72°C, 1 min, for 50 cycles using cheek swab DNA (Richards, B. et al., *Human Molecular Genetics* 2:159-163 (1992)).

15       **Population Survey.** DNA was collected from 87 Scottish terriers from 16 pedigrees. DNA was isolated either from blood using standard procedures (Sambrook, J. et al., Cold Harbor Spring Lab, Cold Harbor Spring NY, 2nd Edition, (1989)) or by cheek swab samples (Richards, B. et al., *Human Molecular Genetics* 2:159-163 (1992)). The genetic status of each animal in the survey was determined  
20 using the *Bst*E I test described above.

## Results

**Comparison of the canine and human sequences.** The alignment of the canine and human prepro-von Willebrand Factor amino acid sequences is shown in Figures 2A-2C (SEQ ID NO: 2). The location of the Scottish terrier vWD mutation is  
25 indicated by the "\*". Potential N-glycosylation sites are shown in bold type. The known and postulated integrin binding sites are boxed. Amino acid numbers are shown on the right side of the figure. The human sequence is derived from Genbank accession number X04385.

Overall, 85.1% sequence identity is seen between the prepro-vWF sequences.  
30 The pro region is slightly less conserved than the mature protein (81.4% vs. 87.5%).

The regions of the gene that are between the known repeat regions within the pro region (data not shown). Fourteen potential N-linked glycosylation sites are present in the canine sequence, all of which correspond to similar sites contained within the human sequence. The two integrin binding sites identified in the human sequence are



sequence (Lankhof, H. et al., *Blood* 86:1035-1042 (1995)) are conserved in the canine sequence as well (Figures 2A-2C; SEQ ID NO: 2). The 5' and 3' untranslated regions have diverged to a greater extent than the coding region (data not shown), comparable to that found between the human and bovine sequences derived for the 5' flanking region (Janel, N. et al., *Gene* 167:291-295 (1995)). Additional insights into the structure and function of the von Willebrand factor can be gained by comparison of the complete human sequence (Genbank Accession No. XO4385; Bonthron, D. et al., *Nucleic Acids Res.* 14:7125-7128 (1986); Mancuso, D.J. et al., *Biochemistry* 30:253-269 (1989); Meyer, D. et al., *Throm Haemostasis* 70:99-104 (1993)) and the complete canine sequence reported here.

The sequence for most of exon 28 was determined (Mancuso, D.J. et al., *Thromb Haemost* 69:980 (1993); Porter, C.A. et al., *Mol Phylogenet Evol* 5:89-101 (1996)). All three sequences are in complete agreement, although two silent variants have been found in other breeds (Table 1, exon 28). Partial sequences of exons 40 and 41 (cDNA nucleotide numbers 6923 to 7155, from the initiation codon) were also determined as part of the development of a polymorphic simple tandem repeat genetic marker (Shibuya, H. et al., *Anim Genet* 24:122 (1994)). There is a single nucleotide sequence difference between this sequence ("T") and the sequence of the present invention, ("C") at nucleotide position 6928.

**Scottish Terrier vWD mutation.** Figure 3 shows nucleotide sequencing ladders for the vWD mutation region for normal (clear), carrier, and affected Scottish terriers. The sequences were obtained directly from PCR products derived from genomic DNAs in exon 4. The arrowheads show the location of the C nucleotide that is deleted in the disease-causing allele. Note that in the carrier ladder each base above the point of the mutation has a doublet appearance, as predicted for deletion mutations. The factor levels reported for these animals were: Normal, 54%; Carrier, 34%; Affected, <0.1%.

As a result of the deletion, a frameshift mutation at codon 88 leads to a new stop codon 103 bases downstream. The resulting severely truncated protein of 119 amino acids does not include any of the mature vWF region. The identity of the base

**Development of a diagnostic test.** A restriction enzyme was designed to create a *Bst*E I site in the mutant allele but not in the normal allele (Figure 4; SEQ ID NOS 3 and 10). The position of the deleted nucleotide is indicated by an asterisk. The restriction enzyme was used to digest genomic DNA from normal and affected

also be distinguished using *Sau96* I. The naturally occurring *Sau96* I sites are shown by double underlines. The highly conserved donor and acceptor dinucleotide splice sequences are shown in bold type.

5 In order to ensure that the restriction enzyme cut the amplified DNA to completion, an internal control restriction site common to both alleles was designed into the non-diagnostic primer. The test was verified by digestion of the DNA from animals that were affected, obligate carriers, or normal (based on high factor levels [greater than 100% of normal] obtained from commonly used testing labs and reported by the owners, and also using breeds in which Type 3 vWD has not been  
10 observed). The expected results were obtained (e.g., Figure 5). Five vWD-affected animals from a colony founded from Scottish terriers (Brinkhous, K.M. et al., *Ann. New York Acad. Sci.* 370:191-203 (1981)) were also shown to be homozygous for this mutation. An additional unaffected animal from this same colony was found to be clear.

15 It would still be possible to misinterpret the results of the test if restriction enzyme digestion was not complete, and if the rates of cleavage of the control and diagnostic sites were vastly different. The rates of cleavage of the two *Bsi*E I sites were thus examined by partially digesting the PCR products and running them on capillary electrophoresis. The rates were found to be very nearly equal (the  
20 diagnostic site is cut 12% faster than the control site).

The mutagenesis primer was also designed to produce a *Sau96* I site into the normal allele but not the mutant allele. This is the reverse relationship compared to the *Bsi*E I-dependent test, with respect to which allele is cut. Natural internal *Sau96* I sites serve as digestion control sites (shown in Figure 4). The test using this  
25 enzyme produced identical genotypic results compared to the *Bsi*E I for all animals examined (data not shown).

**Mendelian inheritance.** One test often used to verify the correct identification of a mutant allele is its inheritance according to Mendel's law of segregation. Three pedigrees were examined in which the normal and mutant alleles were segregating,  
30 as shown in Figure 5. Exon four of the vWF gene was PCR amplified from genomic

... and the test results were as follows: ...  
The affected animals are homozygous for the mutant allele (229 bp; lanes 3 and 5). The other animals in this pedigree are heterozygotes (251 bp and 229 bp; lanes 1

... and ...

**Table 1 - Differences Between Scottie And Doberman Pinscher Protein And Nucleotide von Willebrand Factor Sequences With Comparison To The Human Sequences**

Exon	A.A. <sup>1</sup>	Amino Acid			Codon		
		Human	Scottie	Doberman	Human	Scottie	Doberman
5	5' UT <sup>2</sup>	nuc - 35 <sup>3</sup>	N/A <sup>4</sup>	N/A	N/A	A	G
	4	85	S	S/F Shift <sup>5</sup>	TCC	TCC/TC_	TCC
	5	173	M	R	ATG	AGG	AAG
	11	422	S	T	TCC	ACA	ACC
	21	898	C	C	TGC	TGT	TGC
10	21	905	F	F	TTT	TTC	TTA
	24	1041	S	S	TCA	TCA	TCG
	24	1042	S	S	TCC	TCC	TCA
	28	1333	D	D	GAC	GAC	GAG
	28	1349	Y	Y	TAT	TAT	TAC*
15	42	2381	P	L	CCC	CTG	CCG
	43	2479 <sup>6</sup>	S	S	TCG	TCG	TCA
	45	2555	P	P	CCC	CCC	CCG
	47	2591	P	P	CCC	CCT	CCC
	49	2672	D	D	GAT	GAT	GAC
20	51	2744	E	E	GAG	GAG	GAA

<sup>1</sup>Amino acid residue position

<sup>2</sup>Untranslated region

<sup>3</sup>Nucleotide position

<sup>4</sup>Not Applicable

25 <sup>5</sup>Frameshift mutation

<sup>6</sup>Splice site mutation for Doberman pinscher, Manchester terrier and Poodle

Boxed residues show amino acid differences between breeds

\*This site has been shown to be polymorphic in some breeds

The mature VWF protein begins in exon 18

30 The alleles, as typed by both the *Bst*E I and *Sau*96 I tests, showed no inconsistencies with Mendelian inheritance. One of these pedigrees included two affected animals, the phenotypically normal siblings of both affected animals and one normal sibling of the affected animal. The normal siblings were found to be heterozygotes for the mutation, and the affected animals were found to be heterozygotes.

**Population survey for the mutation** Cheek swabs or blood samples were

collected from 100 dogs of various breeds and genotypes for analysis.

Scottish terrier population. Although an attempt was made to make the sample as random as possible, these dogs were found to come from 16 pedigrees, several of which are more distantly interconnected. This is due to some ascertainment bias, based on ownership (as opposed to phenotypic ascertainment bias). In these 87 animals, 4 affected and 15 carrier animals were found.

### Discussion

These results establish that the single base deletion found in exon four of the vWF gene causes vWD in the Scottish terrier breed. The protein produced from the mutant allele is extremely short and does not include any of the mature vWF protein. Four Scottish terriers known to be affected with the disease are homozygous for the mutation. Five other mixed-breed dogs descended from Scottish terriers, and affected with vWD, are also homozygous for the mutation. No normal animals are homozygous for the mutation. Unaffected obligate carriers are always heterozygous for the mutation.

The gene frequency, as determined from the population survey, appears to be around 0.13 resulting in a heterozygote frequency of about 23% and expected frequency of affected animals of about 2%. Although the sample size is relatively small and somewhat biased, these data are in general agreement with the protein-based surveys (Stokol, T. et al., *Res Vet Sci* 59:152-155 (1995); Brooks, M., *Probl In Vet Med* 4:636-646 (1992)), in that the allele frequency is substantial.

All data collected thus far indicate that this mutation may account for essentially all of the von Willebrand's disease found in Scottish terriers. This result is consistent with the results found for other genetic diseases, defined at the molecular level, in various domestic animals (Shuster, D.E. et al., *PNAS (USA)* 89:9225-9229 (1992); Rudolph, J.A. et al., *Nat Genet* 2:144-147 (1992); O'Brien, P.J. et al., *JAVMA* 203:842-851 (1993)). A likely explanation may be found in the pronounced founder effect that occurs in domestic animals, compared to most human and wild animal populations.

Published data using the protein-based factor assays have shown that, at least in several instances, obligate carriers have had factor levels that would not

have been detected by the protein-based assays (Brinkhous, K.M. et al., *Ann New York Acad Sci* 370:191-203 (1980)). In another study, at least some of the obligate carriers had factor levels of 65% or greater (Brinkhous, K.M. et al., *Ann New York Acad Sci* 370:191-203 (1980)).

substantial. In one study, 19% of Scottish terriers fell in this range (50-65% of the normal vWF antigen level) (Stokol, T. et al., *Res Vet Sci* 59:152-155 (1995)). Thus, although the protein-based tests have been useful, the certainty of the DNA-based test described herein should relieve the necessity of repeated testing and the variability associated with the protein-based assays.

The mutation is present in the pre-vWF part of the molecule. This part of the molecule is processed off prior to delivery of the mature protein into the plasma. This pre-portion of the molecule is important for the assembly of the mature vWF protein (Verwiej, L. et al., *EBMO J* 6:2885-2890 (1987); Wise, R.J. et al., *Cell* 52:229-236 (1988)). With the Scottish terrier frameshift vWD mutation, neither this pre-portion nor any of the mature factor is ever produced, in keeping with the fact that no factor has ever been detected in the blood of affected dogs.

The determination of the complete canine vWF cDNA sequence will have an impact upon the development of carrier tests for other breeds and other species as well. Currently, Shetland sheepdogs (see Specific Example 3) and Dutch Kooikers are known to have a significant amount of Type 3 vWD (Brooks, M. et al., *JAVMA* 200:1123-1127 (1992); Slappendel, R.J., *Vet-Q* 17:S21-S22 (1995)). Type 3 vWD has occasionally be seen in other breeds as well (e.g., Johnson, G.S. et al., *JAVMA* 176:1261-1263 (1980)). All Type 3 vWD mutations described in humans to date have been found within the vWF gene itself. The availability of the canine sequence will make it easier to find the mutations in these breeds. In addition, at least some Type 1 mutations have been found within the human vWF gene, and thus Type 1 mutations may also be found within the vWF gene for breeds affected with that form of the disease. The availability of two divergent mammalian vWF cDNA sequences will also make it much easier to sequence the gene from other mammalian species using cross-species PCR methods (e.g., Venta et al., *Biochem. Genet.* 34:321-341 (1996)).

The test described herein for the detection of the mutation in Scottish terriers may be performed on small amounts of DNA from any tissue. The tissues that are the least invasive to obtain are blood and buccal cells. For maximum convenience, a cheek swab as a source of DNA is preferred.

#### Materials and Methods

**RT-PCR and DNA Sequence Analysis.** RNA was isolated by using Trizol (Life Technologies, Gaithersburg, MD) from the spleen of a Doberman pinscher that was affected with vWD. Total RNA (20 µg) was extracted with Trizol and then treated with

cardiomyopathy. RT-PCR was performed as previously described using primers to the canine vWF cDNA. Most PCR products were determined directly using a cycle sequencing kit (Amersham Corp, Chicago, IL). A minor band containing the four base deletion (see Results) was subcloned into a plasmid vector prior to sequence analysis. The five kb intron 43 was amplified using a commercially available kit for long PCR (Boehringer-Mannheim, Indianapolis, IN). The cycling times and temperatures were as follows: initial denaturation, 93°C, 2 min; 10 cycles of 93°C, 15 sec, 62°C, 30 sec, 68°C, 4 min; 20 cycles of 93°C, 15 sec, 62°C, 30 sec, 68°C, 4 min with 20 additional sec per cycle. This was followed by a final extension at 68°C for 7 min. The sequences of the primers used were: exon 43 (sense primer), 5'-TCTACCCTGTGGGCCAGTTC-3' (SEQ ID NO: 26), and exon 44 (antisense primer), 5'-GACCACCTCACAGGCAGAT-3' (SEQ ID NO: 27).

**PCR-Based Mutation Test.** PCR mutagenesis was used to create an *Msp* I site in the normal allele but not in the mutant allele. An internal *Msp* I digestion control site was also created by PCR mutagenesis within the anti-sense primer, whose target is within intron 43. The control site is contained within the amplification products of both alleles. The sequences of the primers are: diagnostic (sense) primer, 5'-CTGTGAGGACAACTGCCTGCC-3' (SEQ ID NO: 28); and common (anti-sense) primer, 5'-TGGCCCTGAACCGGAATTACTCAAG-3' (SEQ ID NO: 29) (the altered bases within each primer are underlined). A 'touchdown' PCR protocol was used for the amplification. The amplification conditions are: 94°C, 30 sec, 63 to 55°C, 40 sec, and 72°C, 50 sec, for the first 8 cycles, with the annealing temperature dropping one degree per cycle. Twenty-eight additional cycles were run, with the annealing temperature held at 55°C. The DNA was digested with *Msp* I after PCR amplification.

**Population Survey.** Owners who participated in a population survey supplied cheek swabs from their dogs for genotype analysis. Richards, B. et al., *Hum. Mol. Genet.* 2:159 (1992). A number of these dogs had associated vWF values that were determined by various testing laboratories that provide this service to breeders.

## Results

Sequencing of the vWF cDNA revealed a single nucleotide change in the sequence of the vWF cDNA. This change was found at the last base of exon 43 (nucleotide 7437 from the initiation codon, at amino acid position Ser 2479; G in Scotties, A in the affected Bernese Mountain Dogs). Although the change was found in both Scottie and Bernese Mountain Dog breeds, it was only found in the Bernese Mountain Dog breed in the affected dogs.

splice junction to be less similar to the mammalian splice junction consensus. Nakai, K. et al., *Gene* 141:171 (1994); Krawcsak, M. et al., *Genet.* 90:41 (1992). Just upstream of the normal splice junction is another sequence that also has significant similarity to the consensus, which is increased by the A at nucleotide position 7437 (Figure 6; SEQ ID NOS: 14-17). The A at the end of exon 43 could cause the normal splice junction to be used less frequently, and that the upstream cryptic splice site becomes the one predominantly used. Comparison of the splice sites by a devised statistical method (Shapiro, M.B. et al., *Nucleic Acids Res.* 15:7155 (1987)) gave the following scores: normal splice position with the wild-type allele (G at 7536), 83.9; cryptic splice site with the wild-type allele, 60.6; normal splice position with the mutant allele (A at 7437), 72.2; cryptic splice site with the mutant allele, 70.5. Higher scores represent a greater likelihood of splicing potential. The scores for the normal and cryptic splice sites are quite different with the wild-type allele, but are very close with the mutant allele. These results support the probability of a decreased likelihood for splicing at the normal site, and an increased potential for splicing at the cryptic site with the mutant allele.

A faint RT-PCR band just below the major band from which the variant nucleotide had been detected was observed. This minor band was missing the four bases at the end of exon 43 as confirmed by sequence analysis (Figure 7; SEQ ID NO: 18). The position of the four deleted bases is shown on the right side of Figure 7 (SEQ ID NO: 18).

A PCR-based test was developed to detect the nucleotide difference in genomic DNA as described herein in Materials and Methods. The results of the test for several animals with a spectrum of factor values yield a significant correlation between genotype and factor value as shown in Figure 8. Lane 1 contains a 50 bp ladder as a size marker. The uncut PCR product is 135 bp (lane 8). Both alleles contain a common Msp I restriction site that serves as an internal digestion control. The mutant (A) and normal (G) alleles are represented by the 123 bp and 102 bp bands, respectively. Reported factor levels and deduced genotypic status for dogs represented in the additional lanes are as follows: 2, 12 %, affected (AA); 3, 8 %,

ascertained animals with associated factor values showed a strong correlation between genotype and factor level as presented in the histogram of Figure 9. The

consistent with the genotypes deduced from the PCR-based diagnostic test. Larger factor value-only surveys (Johnson et al., *Vet. Clin. North Am. Small Anim. Pract.* 18:195-229 (1988); Moser et al., *Am. J. Vet. Res.* 57:1288-1293 (1996); Stokol et al., *Aust. Vet. J.* 72:257-262 (1996)) indicate substantial overlap between genotypes based upon the protein-based methods. A larger survey on 67 additional Dobermans contained in 10 independently ascertained pedigrees was performed to obtain an estimate of the mutant allele frequency within the breed. Of the total of 88 animals, 40 were AA, 35 were AG, and 13 were GG. From these results, the A allele frequency was estimated to be 0.64.

### Discussion

The splice junction mutation at the end of exon 43 is the cause of recessive Type 1 vWD found within the Doberman pinscher breed. The mutation decreases the similarity between the normal splice junction and the mammalian consensus while at the same time increasing the similarity of the cryptic splice site found just upstream of the normal splice site (Figure 6; SEQ ID NOS: 14-17). The calculated Shapiro-Senapathy splice site values (Shapiro, M.B. et al., *Nucleic Acids Res.* 15:7155 (1987)) are very similar for the normal and cryptic splice sites when an A is present at nucleotide position 7536. The Shapiro-Senapathy calculation is probably not completely accurate in determining the relative amount of splicing that can occur between different sites. Therefore, it is not inconsistent to find that the cryptic splice site is used more often than the normal site, in the mutant allele.

The sequence of the minor amplification product seen just below the main amplification band exactly matches that predicted by the use of the cryptic splice site (Figure 7; SEQ ID NO: 18). The fact that there is less cryptically spliced mRNA than normally spliced mRNA present in the cytoplasm can be explained by the relative instability of the cryptically spliced message. The cryptically spliced mRNA produces a shift in the translational reading frame, resulting in the formation of a premature stop codon. It is well known that mRNAs that produce truncated proteins are unstable, perhaps because ribosomes do not remain attached to the message to protect it from

the average amount of vWF protein present in the blood plasma is roughly 10% of the normal canine value. Thus, each mutant allele should produce about 5% of the normal amount of vWF mRNA and protein. From this, it can be predicted that the average heterozygote (AG) should produce about 5% of the normal amount of vWF



value. The vWF mRNA estimated in affected animals has been shown to be roughly 20% of normal by densitometry scans of northern blots. Meinkoth, J.H. et al., *Am. J. Vet. Res.* 56:1577 (1995). This mRNA is predicted to consist primarily of the correctly spliced transcript.

5           The mutation has been shown to be linked to the vWF locus (Figure 9 and Holmes, N.G. et al., *J. Small An. Prac* 37:307 (1996). Most human Type 1 vWD, in which there is a true clinical bleeding problem, appears to be inherited in a dominant, incompletely penetrant fashion. Ginsburg, D. et al., *Blood* 79:2507 (1992). Although a few Type 1 mutations have been found within the vWF locus (see, e.g., Siguret, V. et al., *Hum. Genet.* 93:95 (1994); Eikenboom, J.C.J. et al., *Blood* 88:2433 (1996)), it has been argued that another locus or loci may also cause some Type 1 vWD. Ginsburg, D. et al., *Blood* 79:2507 (1992). In fact, one murine Type 1 vWD has been mapped to locus that is not linked to the vWF gene. Nichols, W.C. et al., *Blood* 83:3225 (1994). The data show that a least a proportion of Type 1 vWD in humans  
10           might also be caused by the exon 43 mutation, or other leaky splice junction mutations. The mode of inheritance for this type of mutation is recessive, but it might appear to be dominant in certain situations, such as that of the Doberman pinscher. The number of splice site mutations of the type described herein are significantly below the number that would be predicted to occur, suggesting that these types of  
15           mutations are more difficult to detect or have been overlooked in the past. Krawcsak, M. et al., *Hum. Genet.* 90:41 (1992). This might be because they produce a less severe phenotype than other types of mutations that cause a complete loss of function.

### **SPECIFIC EXAMPLE 3 - SHETLAND SHEEPDOG**

25           Total DNA was isolated from material obtained from a spay of an affected Shetland sheepdog (Sheltie). This animal had been tested for the vWF antigen, and was reported to have a 0% value by a laboratory skilled in this testing (Diagnostic Laboratory, Comparative Hematology Section, College of Veterinary Medicine, Cornell University). The owner had decided to have the spay done after obtaining this result,  
30           and donated the removed tissues. The entire RT-PCR coding region of this mutant

      vWF cDNA (mutant) was found to differ from the normal cDNA (GenBank#U05509) for most or all of the type 3 vWD found in the Sheltie breed. A deletion of a single T was found at nucleotide position 735 of the encoding region (Figure 10; SEQ ID No. 10). This mutation is shown in Figure 10 and also the sequence of the mutant.

which one T has been deleted in the DNA of the affected animal compared to the normal animal. This deletion, present in the equivalent of human exon 7, would cause a shift in the reading from of the vWF-encoding region, and result in a severely truncated protein. A diagnostic test was designed to detect this mutation (Figure 11; SEQ ID NOS: 21-25). The deletion causes the creation of an *Mwo* I restriction site and thus, the *Mwo* site is found in the mutant allele, but not in the normal allele. The sequence shown in Figure 11 (SEQ ID NOS: 21 and 22) is that of the canine gene that corresponds to the human vWF exon 7. The single letter code for amino acids is shown above the nucleotide sequence and the primer sequences are shown below the gene sequence. The *Mwo* I sites are also indicated. An internal digestion control site is present in the non-diagnostic primer region. Reagent concentrations for this test were: 100  $\mu$ M dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM  $MgCl_2$ , 0.05 to 0.1  $\mu$ g target DNA, 15  $\mu$ M of each primer (SEQ ID NOS: 23 and 25), and 0.025 U Taq DNA polymerase. Cycling conditions were: 94°C, 4 min, one cycle, followed by 50 cycles of 94°C, 30 sec, 63°C, 40 sec, and 72°C, 40 sec. The relatively low Taq concentration (compared to generally accepted conditions) with the high number of cycles prevents the amplification of non-specific PCR bands. One microliter of *Mwo* I restriction enzyme (New England Biolabs, Inc.) and 2  $\mu$ l of 50 mM  $MgCl_2$  were added directly to the PCR reaction after amplification, and incubated at 60°C for 1 hr. Digestion products were then observed after gel electrophoresis on a 1.5% agarose gel and the results shown in Figure 12. Lanes 1 and 17 show a one hundred bp ladder. Lanes 2-6 show the results from an affected animal, lanes 7-11 show the results from a carrier animal, and lanes 12-16 show the results from a homozygous normal animal. Lane 18 shows an undigested control PCR product. The duplicate samples demonstrate the reproducibility of the test. Numbers on the left side of the gel show the sizes of the standard bands, and numbers on the right side of the gel show the sizes of the uncut product (U), the normal allele (N), and the two bands for the mutant allele (M).

A survey of Shelties was conducted to determine the frequency of the mutation within the U.S. population. Of a total of 103 animals, 14 were carriers, giving a carrier

*J. Am. Vet. Med. Assoc.* 200;1123-1127 (1992). One third of these carriers are thought to be due to Type 1 vWD also present in the breed. Still, the value of 13.6% is not statistically different from the value of 13.6% reported by the author.

difference could be due to either ascertainment biases in either study, a true decrease in the frequency of the disease in this breed, one or more additional Type 3 mutations in the breed, or a combination of these possibilities. Whatever the reason for the difference, most or all of the Type 3 disease in the Sheltie is probably caused by this one mutation. This is based on the understanding of the importance of the Founder effect (or populate sire effect) on the increase in the frequency of specific genetic diseases in purebred populations of domestic animals. A 17 member pedigree of Shelties, in which the mutation was segregating was tested for normal Mendelian inheritance of the allele. There were no differences from what would be expected under co-dominant inheritance of the two alleles.

#### SPECIFIC EXAMPLE 4

In an effort to find mutations that cause vWD in other canine breeds, affected animals were surveyed, as diagnosed by low levels of vWF antigen, for the three mutations set forth herein. In the case of the Manchester terrier breed, it was found that at least a portion of the affected animals had the identical mutation that causes vWD in the Doberman pinscher. The test described *supra* for the Doberman pinscher was utilized to test an affected Manchester terrier, plus several related animals. The affected animal was found to be homozygous for the mutant allele (Table 2). In addition, several animals who had vWF values in the carrier range were found to be carriers at the genotypic level.

Table 2

Manchester terrier vWF values vs. DNA genotype

Dog	vWF value <sup>a</sup>	Genotype <sup>b</sup>
MT1	200%	normal
MT2	76%	normal
MT3	42%	carrier
MT4	19%	carrier
MT5	NT	carrier
MT6	NT	carrier

<sup>a</sup>Genotype for the leaky splice mutation originally found in the Doberman pinscher.

### SPECIFIC EXAMPLE 5

In an effort to locate mutations that cause vWD in other canine breeds, affected animals as diagnosed by low levels of vWF antigen, were surveyed for the three mutations set forth herein. The test described *supra* for the Doberman pinscher  
5 was utilized and, in the case of the Poodle breed, it was found that the affected animals had the identical mutation that causes vWD in the Doberman pinscher. The affected animals were found to be homozygous for the mutant allele. In addition, several animals who had vWF values in the carrier range were found to be carriers at the genotypic level.

10 The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying drawings, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention.

15 All patents and other publications cited herein are expressly incorporated by reference.